Toward Flow Cytometry Based Platelet Function Diagnostics

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Abstract

The laboratory diagnostics of (inherited) platelet function disorders mainly comprises aggregation and secretion assays, which may be suitable for diagnosing some specific severe platelet function disorders, but are not reliable enough for diagnosing mild platelet function disorders or disorders associated with low platelet count. Flow cytometric assessment of platelet reactivity will expectedly provide additional value during the diagnostic work-up of platelet function disorders because it only requires a small volume of whole blood and allows the measurement of platelet function in thrombocytopenic samples. Flow cytometry has frequently been used to evaluate platelet function in the research setting, and therefore, these assays will require clinical validation before they can be used as routine diagnostic tools. The main challenge in the validation of innovative platelet function diagnostic tests is the lack of a gold standard test for mild platelet function disorders. This review aims to address the many applications of flow cytometry in the current diagnostic work-up of platelet function testing and to discuss the challenges in introducing new tools for diagnosing platelet function disorders.

Keywords
- flow cytometry
- blood platelets
- blood platelet disorders
- platelet function tests

Platelets are the main players in response to vascular injury and for preventing excessive blood loss. These small blood elements rapidly adhere to the site of injury and form a hemostatic plug to limit abnormal blood loss. Platelet function in hemostasis is roughly divided into four different processes, namely, platelet adhesion, activation, secretion, and aggregation. The first step in platelet adhesion is the binding of the glycoprotein (GP) Ib-V-IX complex to immobilized von Willebrand factor (VWF).[1] The subsequent interaction of α2β1 and GPVI with subendothelial collagen, along with integrin αIIbβ3 (previously named GPIIb/IIIa) binding to VWF, results in platelet activation and firm adhesion.[2] Platelet activation results in α and dense granule secretion, generation of thromboxane A2, and αIIbβ3 activation. The secretion of α and dense granules, along with the production of thromboxane A2, further promotes platelet activation, whereas activation of αIIbβ3 results in fibrinogen binding to crosslink activated platelets.

Platelet function disorders (PFDs) are characterized by easy or spontaneous bruising, mucocutaneous bleeds, or prolonged bleeding time and have a heterogeneous phenotype.[3] They can be inherited or acquired and can be associated with a low platelet number (i.e., thrombocytopenia).[4,5] Qualitative PFDs include Glanzmann's thrombasthenia (GT), storage pool disease (SPD), and Bernard–Soulier's syndrome (BSS). Other PFDs include Gi-receptor disorders, collagen receptor disorders, and secretion disorders.[6]

An accurate diagnosis is vital for quality of life and prevention of life-threatening bleeding episodes with prophylactic therapy. It is also essential to distinguish between congenital platelet deficit and other forms of thrombocytopenia, such as immune thrombocytopenia. The laboratory diagnostics of

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PFDs entails many different techniques. The currently available diagnostic tools may be suitable for identifying severe PFDs, especially GT; however, identification of mild PFDs including SPD and BSS remains challenging. There is therefore an unmet need for improved platelet function diagnostics, especially in thrombocytopenic samples. Flow cytometry appears suitable for assessing platelet function in samples with low platelet count and has been suggested as a viable alternative to currently available tools. However, flow cytometry needs both validation for mild PFDs and standardization before it can be used in a routine diagnostic setting. In this review, we will discuss the current diagnostic strategy for PFDs, the potential role of flow cytometry in platelet diagnostics, and the challenges of introducing new diagnostic tests for PFDs.

Diagnostic Work-Up of Suspected Platelet Function Disorders

The first step in the diagnosis of PFDs is based on the evaluation of symptoms, bleeding, and family history (►**Fig. 1**). The accurate assessment of a bleeding disorder encompasses the possible use of standardized questionnaires, also known as bleeding assessment tools (BATs), which allow quantification of the severity and frequency of bleeding episodes, finally expressed as a bleeding score. A commonly used bleeding score is the Tosetto score, which has been designed for the assessment of patients with von Willebrand's disease (VWD).[7] A score of ≤ 3 almost completely rules out VWD, exhibiting a negative predictive value (NPV) as high as 99%.[8] Conversely, a bleeding score of >3 suggests that laboratory testing for a bleeding disorder should be initiated. Nevertheless, the Tosetto score has not been validated for PFDs. The International Society on Thrombosis and Haemostasis (ISTH) has developed an alternative bleeding score, the ISTH-BAT, that can be used for both VWD and PFDs.[9] The ISTH-BAT bleeding score also displays a high NPV but is characterized by low specificity and positive predictive value.[10] The normal score is ≤ 3 for men and ≤ 5 for women.[11]

Before performing specific platelet function analyses, screening tests should be performed to confirm a suspicion of PFDs and to exclude VWD and coagulation factor deficiencies. In case of a high bleeding score and exclusion of VWD or coagulation factor deficiencies, a PFD is very likely.[12] The Platelet Function Analyzer-100 or 200 (PFA-100/200) is a sensitive analyzer for screening patients with primary hemostasis disorders but is not sensitive enough for distinguishing PFDs from VWD.[13] A complete blood cell count, including platelet number, mean platelet volume, and number of reticulated platelets, can help identify and differentiate thrombocytopenias. For example, the number of reticulated platelets may help distinguish between disorders characterized by decreased platelet production or enhanced platelet turnover.[14] A peripheral blood smear can also be helpful in diagnosing May–Hegglin's disease, gray platelet syndrome, or pseudothrombocytopenia (e.g., due to EDTA [ethylenediaminetetraacetic acid]).[15,16]

Among all different platelet function tests, the most frequently used test for the diagnosis of PFDs is light transmission aggregometry (LTA).[17] A reliable alternative is whole-blood impedance aggregometry, which is less time-consuming and can be performed in whole blood but still requires more validation for use in the diagnostic workout of inherited

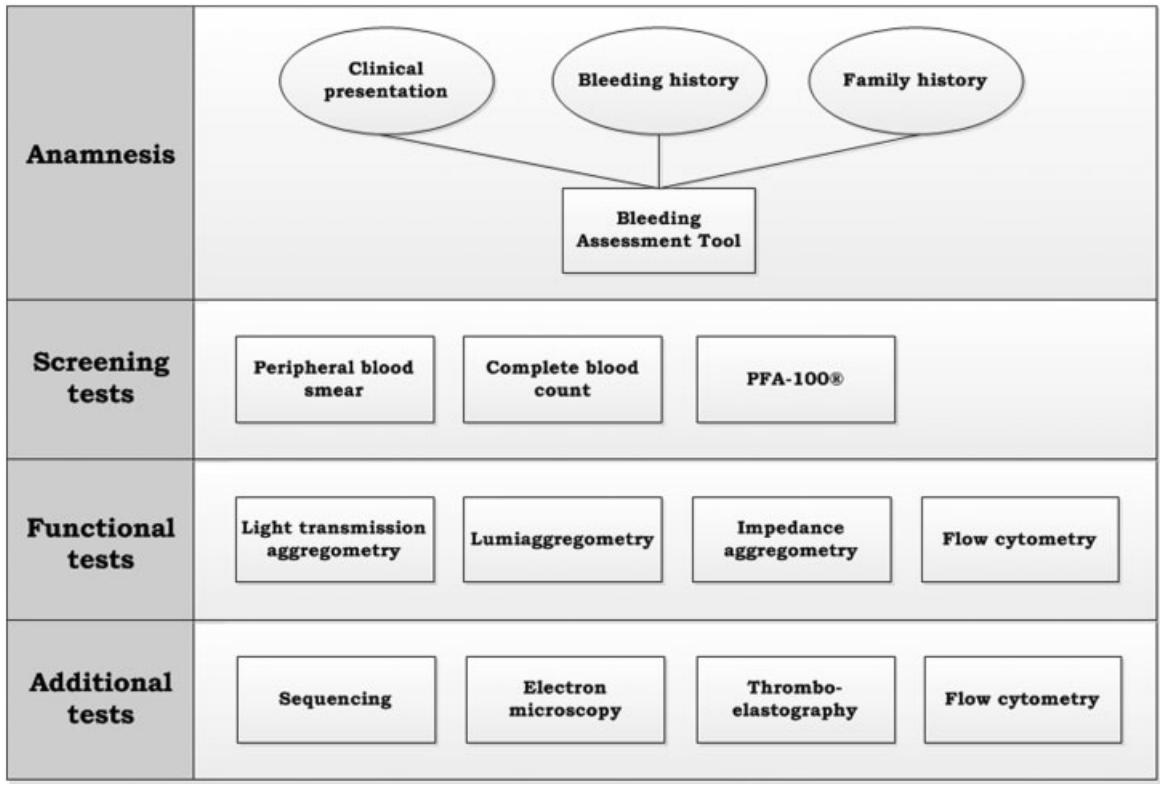

Fig. 1 The diagnostic flowchart of platelet function testing.

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PFDs.[18] LTA measures the light transmitted through platelet-rich plasma (PRP) after stimulation with different agonists, which are used in several concentrations. Upon platelet stimulation, light transmission through the sample increases over time as a result of platelet aggregation and can hence be considered a reliable measure of platelet function. Ristocetin-induced platelet agglutination is a measure of platelet adhesion. The most frequently used parameters include maximum aggregation amplitude, final amplitude, and curve reversibility. By using a different subset of agonists, different platelet activation pathways can be studied. The most commonly used agonists are adenosine diphosphate (ADP), collagen, arachidonic acid, epinephrine, and ristocetin.[19] The diagnosis of GT and BSS can only be made with LTA, although some cases may be missed due to low platelet count. Impaired aggregation after stimulation with all platelet agonists except ristocetin is observed in GT, whereas normal platelet aggregation with only impaired ristocetin-induced platelet agglutination is found in BSS. Not all patients with SPD display decreased platelet aggregation with LTA, which explains why SPD cannot be ruled out when LTA is normal.[20,21] Therefore, the diagnosis of SPD requires additional testing. The diagnosis of mild heterogeneous PFDs with only LTA becomes even more challenging. Although LTA is an important test for diagnosing different severe PFDs, this test lacks sensitivity for the diagnosis of mild PFDs.[6] The overall sensitivity of LTA for PFDs is estimated between 34 and 60%,[22] which is probably an underestimation due to the underdiagnosis of mild forms of PFDs.[12] Moreover, a major drawback of LTA is that it is not feasible, or reliable, to investigate platelet function in thrombocytopenic samples. Nevertheless, measuring platelet function in thrombocytopenic samples is vital since a platelet count of $<50 \times 10^9$/L is not necessarily associated with a bleeding diathesis.[23] According to general consensus, the minimum platelet count of 150×10^9/L platelets is necessary for obtaining reliable LTA data.[24] The assessment in samples with a lower platelet count is still feasible but should be interpreted with caution, especially when the platelet count falls below 75×10^9/L platelets.[25] In addition to the mild sensitivity for PFDs, LTA exhibits additional drawbacks. LTA is poorly standardized, and there is poor consensus about the concentration and number of agonists, thus explaining the poor interlaboratory agreement.[17,26] The recommended panel of agonists for LTA is extensive since it is necessary to gather the larger possible information on platelet function. LTA is hence labor-intensive and time-consuming and requires a high volume of whole blood, which often precludes the diagnosis of PFDs in neonates and young children.[27]

Due to drawbacks and lack of sensitivity of currently available tests, we need new methods for diagnosing PFDs, preferably encompassing collection of a minimal amount of blood. Flow cytometry has been recommended by the ISTH Scientific and Standardization Committee as a reliable diagnostic tool for diagnosing PFDs.[6] The main advantage of flow cytometry over routine platelet function tests is the minimum amount of whole blood needed and its possible use in patients with thrombocytopenia.[28,29] The use of whole blood makes this technique less time-consuming and less vulnerable to preanalytical problems. However, flow cytometry also has drawbacks, just like LTA; shear stress forces are not taken into account during the analysis of platelet function and reagents are relatively expensive. Moreover, flow cytometry is only available in a minority of diagnostic laboratories as a first-line laboratory test.[12] The current role of flow cytometry in PFD diagnostics entails measuring surface receptor expression to confirm the diagnosis of BSS or GT, but can also use be used to test additional receptors, such as αIIβ1, GPIV, and GPVI, for diagnosing collagen receptor deficiencies.[6] Moreover, agonist-induced platelet reactivity can be measured with a flow cytometer, but is mainly used in experimental settings. It lacks validation and standardization to broaden its use in routine diagnostics so far.

Flow Cytometric Testing of Platelet Function

Practical Issues

Flow cytometry allows the rapid analysis of multiple cell markers on single cells with fluorescent antibodies and has several advantages compared with LTA (►**Table 1**). The use of multiple excitation lasers and excitation or emission filters allows detecting different platelet markers within one sample after compensation of fluorescent intensity. To identify platelets by flow cytometry in whole-blood samples, PRP, or washed platelets, logarithmic scaling of forward scatter (FSC) and sideward scatter (SSC) is needed. With these settings, normal sized platelets are easily separated from red blood cells (RBCs) and leucocytes, thus allowing analysis of specific receptors or activation markers with fluorescent dyes or antibodies. When platelet identification is only based on FSC and SSC, caution should be taken to avoid an analysis of hemolytic specimens, samples with a large amount of cell debris, or samples with air bubbles, as these will partly overlap with platelets. It should be noted that the FSC/SSC characteristics of large platelets, as found in patients with macrothrombocytopenia, overlap with those of other blood cells, thus hampering accurate platelet identification (►**Fig. 2A**). To prevent these issues, antibodies against a platelet-specific marker, such as GP1bα or CD41, should be added to help distinguish platelets from RBCs or cellular debris.[30]

Antibodies against platelet activation markers can be added to the test samples to assess response to stimulation with agonists. Whole blood should be diluted >1:10 to prevent generation of platelet aggregates.[31] Rigorous sample mixing (e.g., on a vortex mixer) should be avoided to prevent platelet activation and aggregation.

Sample analysis cannot be performed immediately since samples should be fixed after incubation with agonists or antibodies against surface markers. Fixation can be performed with either formaldehyde, glutaraldehyde, or methanol solutions, but standardized fixation reagents are also available. The main advantages of fixation are that samples can be collected prior to analysis, samples can be transported to centers where flow cytometry is available, and further ex-vivo activation events can be avoided.

Platelet function can be assessed in terms of either percentage positive events or median fluorescent intensity (MFI). The "percentage positive event" parameter is only useful when the

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Table 1 Advantages and disadvantages of flow cytometry and LTA

	Flow cytometry	LTA
Advantages	Whole-blood test	Measures platelet function over time
	Requires low amount of whole blood	
	Allows platelet reactivity testing and measurement of surface receptor expression	
Disadvantages	Expensive	Requires large amount of whole blood
	Difficult to standardize	Not possible in samples with low platelet counts
	Requires validation	Labor-intensive
	Only end-point measurements	Time-consuming
	Does not measure platelet function under shear stress	Many preanalytic variables
		Difficult to standardize
		Does not measure platelet function under shear stress

Abbreviation: LTA, light transmission aggregometry.

threshold for positivity is based on unstimulated cells since preactivated platelets already express activation markers such as P-selectin. To sidestep underestimation of positive cells, thresholds should be determined in a sample with an isotype control antibody of the same immunoglobulin class conjugated with an identical fluorophore. The "percentage positive event" parameter is particularly useful when activation markers are assessed in samples in which part of the population is not expected to express this marker. The major advantage of this method is that these semiquantitative data are exchangeable between different laboratories because the settings of flow cytometer and the evaluation software have a modest influence on test outcome. Nevertheless, the drawback is that the percentage positive platelet parameter does not provide information about the degree of activation above the arbitrarily chosen negative threshold. Fully quantitative measures, such as MFI, provide more information on the degree of activation (►**Fig. 2B**) and are therefore more suited to quantify receptor expression than percentage positive events. The major drawback of the MFI is its dependence on the type of flow cytometer, laser quality and alignment, sensitivity, and compensation settings of the flow cytometer, as well as the analysis software. This makes it difficult to standardize flow cytometry and compare MFI among different laboratories. In diagnostics or long-term clinical studies, changes in laser quality and batch-to-batch variations could also introduce unwarranted variation. An internal quality control is advisable to achieve good reproducibility and allows a major degree of interlaboratory exchangeability.

Platelet Reactivity Testing

Platelet function testing should include the assessment of the most common platelet signaling pathways leading to platelet activation. These include the collagen pathway, stimulated through GPVI, the thrombin pathway, in which protease-activated receptor 1 (PAR-1) and PAR-4 play a role, the ADP

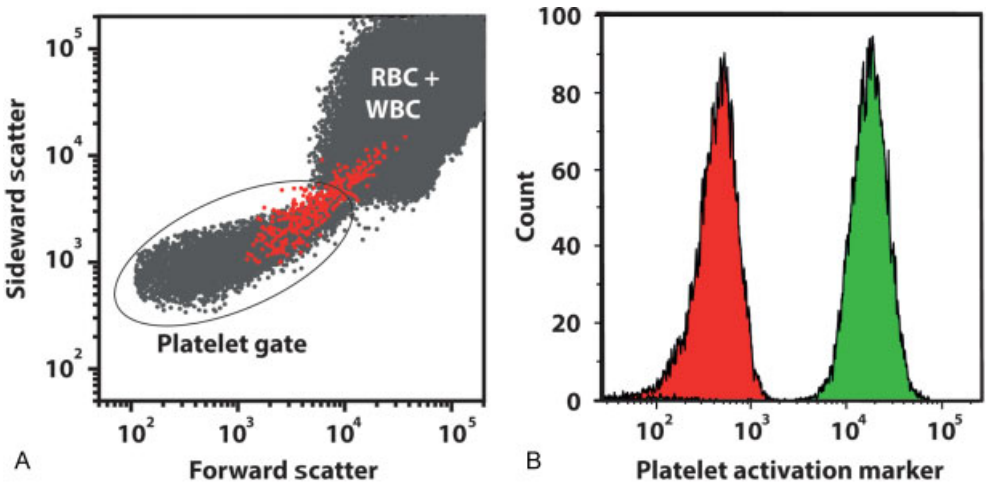

Fig. 2 Whole-blood platelet function testing on the flow cytometer. **(A)** Typical plot of a 10-fold diluted whole-blood sample (gray) on the flow cytometer. Platelets from healthy controls are identified based on forward scatter and sideward scatter using a platelet gate. Large platelets from a patient with macrothrombocytopenia (red; mean platelet volume of 20 fL) are hidden behind red blood cells (RBCs) and white blood cells (WBCs). **(B)** The median fluorescent intensity of a platelet activation marker in resting platelets (red) shifts to the right (green) in response to platelet stimulation.

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pathway, in which the role of the purinergic receptor P2Y12 is deemed very important, and the thromboxane A2 pathway. These pathways can easily be assessed with a standardized agonist panel, similar to that used in LTA, thus including ADP and peptide agonists for the thrombin receptors (PAR1-AP and PAR4-AP). However, the preferred agonist to test the collagen pathway in flow cytometry differs from the most commonly used source of collagen used in LTA. While fibrillar equine collagen type I (Horm collagen) is commonly used in LTA, this agonist is unsuitable for flow cytometry due to its fibrillar structure, which induces formation of platelet aggregates. Crosslinked collagen-related peptide is hence preferable in flow cytometry.[32] Another difference between LTA and flow cytometry is the relative insensitivity of platelets to exogenous arachidonic acid when platelets are not stirred, thus precluding direct appraisal of cyclooxygenase-1 (COX-1) activity with flow cytometry. Synthetic thromboxane A2 analogues, such as U46619, can be used to assess thromboxane A2 mediated platelet activation.

The optimal agonist concentration for assessing platelet reactivity differs among laboratories and depends on the assay purpose. For example, in case of platelet function testing for bleeding disorders, a high agonist concentration is needed to detect decreased platelet reactivity.[33] Since flow cytometry allows a fully quantitative analysis of platelet reactivity based on median fluorescent activity, which cannot be made with semiquantitative LTA, the true activation potential of platelets can be determined. On the other hand, a lower agonist concentration or a concentration gradient is more suitable in the presence of increased platelet reactivity.

Platelet reactivity can be measured using platelet activation markers (►**Fig. 3**), especially including those targeting or reflecting αIIbβ3 activation and granule secretion.[28,34] The measurement of αIIbβ3 activation can be performed with PAC-1 antibody or by measurement of fibrinogen binding.[35] PAC-1 binds the activated αIIbβ3 receptor but fails to provide information on actual fibrinogen binding. On the other hand, fibrinogen binding assessment does not allow to distinguish between impaired αIIbβ3 function and fibrinogen deficiency.

Platelet Secretion Testing

The enumeration of dense granules per platelet using whole mount electron microscopy is the gold standard for diagnosing SPD.[36] Unfortunately, this technique is only available in a few laboratories and is time-consuming. The most commonly used tests are based on luminescence measurement of platelet ADP and adenosine triphosphate (ATP) levels in platelet lysate[37] or on lumiaggregometric assessment of ATP secretion during platelet aggregation.[38] The assessment of ADP concentration in platelet lysate is a measure of potential platelet secretory capacity,[39] whereas lumiaggregometry only measures ATP but not ADP secretion. Both tests cannot be performed in thrombocytopenic samples and also encompass collecting a large volume of blood.

With flow cytometry, platelet secretion markers can be measured upon platelet stimulation. P-selectin, CD63 expression, and lysosomal-associated membrane protein 1 (LAMP-1) are intracellular markers expressed on platelet surface upon platelet activation. They can hence be used as markers of both platelet activation in general and, more specifically, platelet secretion.[40–42] Although all of these proteins are present in intracellular granules and are exposed after activation, they are not completely interchangeable, as they provide information on different granule types. P-selectin, for example, is located in both α and dense platelet granules,[43] whereas CD63 is only present in the dense granules and is hence a better marker for diagnosing SPD.[44] A low CD63 expression can result from impaired platelet activation, secretion, or absence of platelet dense granules and is thus not specific enough for diagnosing SPD. LAMP-1 is a lysosomal and dense granule specific protein, which is secreted upon activation.[45]

The assessment of mepacrine uptake in resting platelets is an alternative approach for flow cytometric quantification of dense granule content. Mepacrine is a fluorescent acridine

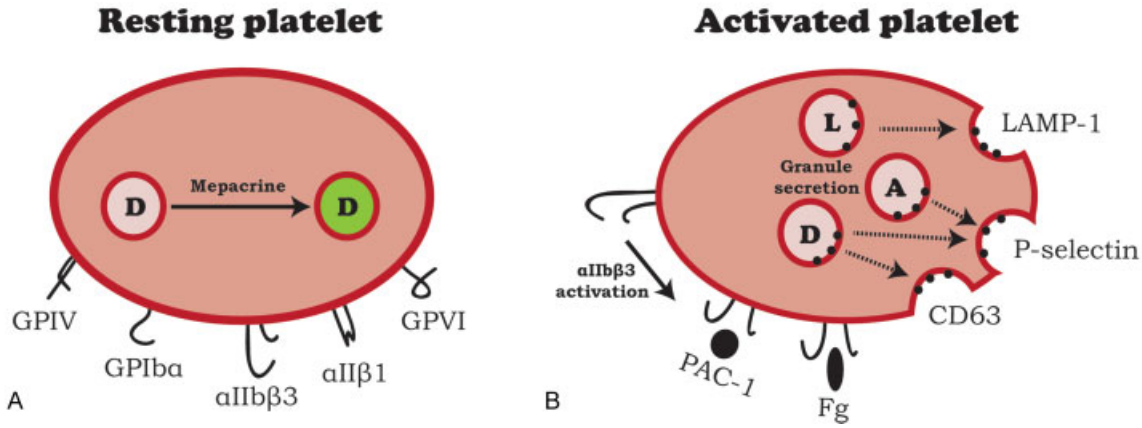

Fig. 3 Markers for platelet function. **(A)** In resting platelets, the most abundant platelet receptors can be quantified. These receptors include glycoprotein (GP) IV, GP1bα, αIIbβ3, αIIβ1, and GPVI. Also, the adenosine diphosphate content in dense granules (D) can be quantified using the fluorescent acridine derivative mepacrine. **(B)** After platelet activation, integrin αIIbβ3 activation results in the binding of PAC-1 or fibrinogen (Fg). Upon platelet stimulation, dense granules, α granules (A), and lysosomes (L) will fuse with the outer membrane and platelet secretion markers, including CD63, P-selectin, and lysosomal-associated membrane protein 1 (LAMP-1), can be quantified.

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derivative with high affinity for adenine nucleotides, which are primarily stored in platelet dense granules. Therefore, mepacrine-labeled dense granules may allow their quantification with flow cytometry. Although mepacrine uptake is low in patients with SPD, this technique has not been validated and its diagnostic performance has not been compared with that of other SPD-specific diagnostic tests.[46,47]

In addition to these classical markers of platelet activation, flow cytometry can be used to assess the expression of anionic phospholipids on activated platelets, a method also known as platelet procoagulant activity. Anionic phospholipids, such as phosphatidylserine (PS), are essential for propagation of coagulation reaction, and platelets are considered the main source of these phospholipids at the site of injury. In Scott's syndrome, platelets lack the membrane phospholipid scramblase necessary for PS expression, thus resulting in decreased procoagulant activity and bleeding diathesis.[48] Procoagulant activity can be measured with fluorophore-conjugated annexin A5,[49] which specifically binds to PS in the presence of Ca^{2+} ions, or lactadherrin,[50] a calcium-independent PS-binding protein.

Finally, ristocetin-induced VWF binding to platelet can be measured in LTA to assess GP1b function. This can be mostly used for investigating BSS, type 2B VWD, or platelet-type VWD. The ristocetin-induced VWF binding assay can also be performed with flow cytometry to assist the diagnosis of VWD or BSS.[51,52] Notably, quantification of GP1b receptor remains the gold standard for diagnosis BSS.

Other Applications of Flow Cytometry Based Platelet Function Testing

Beside the potential application of flow cytometry in diagnosing bleeding disorders, there are other platelet-related applications in which flow cytometry may be useful.

One of these applications is in transfusion medicine. Long-term storage of platelet concentrates causes platelet storage lesion, characterized by decreased platelet function and viability.[53] The in vitro quality of platelet transfusion concentrates is therefore frequently assessed using flow cytometry. The expression of platelet surface markers such as GP1bα and αIIbβ3 can be investigated to monitor platelet quality,[54] but the expression of P-selectin or CD63 in resting platelets is also used to assess preactivated platelets.[55] PS exposure can be determined through annexin V binding or lactadherin to identify the amount of apoptotic platelets in stored platelet concentrates.[54] Nevertheless, the correlation of these markers with in vivo viability and function of platelets remains poor.[56] The residual platelet activation potential, or the agonist-induced platelet reactivity of residual platelet activation potential, may better predict in vivo viability of stored platelets.[57] However, this approach has not been validated so far.

Although thrombotic risk estimation and antiplatelet drug monitoring are other potential applications of flow cytometry in platelet function testing, the clinical usefulness of platelet function testing in patients receiving antiplatelet drugs remains uncertain. The vasodilator-stimulated phosphoprotein (VASP) assay is an already available flow cytometric assay for measuring P2Y12 function and quantifying the phosphorylation state of VASP after ADP stimulation.[58] A good correlation was demonstrated with LTA after in vitro P2Y12 inhibition,[59] but a poor correlation was instead observed for measurement of antiplatelet efficacy in patients receiving anti-P2Y12 therapy.[60]

Validation of Flow Cytometric Assay Is Challenging

The validation of a flow cytometry based test using clinical presentation as the gold standard is unfeasible. The hemostatic system is complex, and its disorders are associated with overlapping clinical signs and symptoms. Only based on signs and symptoms, it is hence challenging to classify bleeding disorders into those affecting vascular wall integrity, PFDs, VWD, coagulation factor deficiencies, or fibrinolytic dysfunction.[61] A validated bleeding score like the ISTH-BAT can be useful to assess whether a patient has an increased bleeding risk, but the results of the bleeding scores should always be interpreted with caution. Almost 25% of young healthy women report at least two bleeding symptoms, thus generating a high bleeding score in the absence of clinically significant bleeding.[62] Moreover, a high ISTH-BAT bleeding score does not allow to differentiate between PFDs and VWD and cannot be used to classify a particular type of PFD. Therefore, the ISTH-BAT is mainly used as a screening tool to determine whether additional laboratory testing is necessary. When diagnostic tools do not display sufficient sensitivity and specificity, the distinction between platelet dysfunction and other defects is challenging.

There is no single gold standard test for PFDs. Although LTA is the most commonly used platelet function test and is an important diagnostic aid, it cannot be used to diagnose all PFDs. LTA measures agonist-induced aggregation, and the test is hence sensitive to both functional defects and platelet quantity. Due to this limitation, LTA is relatively insensitive to diagnose BSS, which is frequently associated with thrombocytopenia. Moreover, patients with an overt bleeding diathesis due to SPD might display normal platelet aggregation measured with LTA.[20,63] Additional diagnostic tools, such as flow cytometry or platelet nucleotide content, are necessary in these cases to make an accurate diagnosis.

The biggest challenge in validating a new platelet function test, such as flow cytometry, is defining the patient population. Without a gold standard, which can be a single test or a combination of several tests, it is impossible to determine whether an abnormal flow cytometric measurement should be considered a true-positive or a false-positive. An ideal population to validate new diagnostic tests consists of a patient group in which the presence of the disorder can be confirmed or excluded based on a gold standard (reference) test. SPD is an example of a primary hemostasis disorder in which these studies are possible because the diagnosis SPD is based on platelet ADP content, for which well-defined and reproducible tests are available.[6] In a SPD population, the diagnostic accuracy of both LTA and flow cytometry can be compared with the gold standard (i.e., platelet ADP content). Nevertheless, SPD is only one example of the many different causes of PFDs and represents a minority of all patients with a defect of primary hemostasis. The majority of patients presenting with a bleeding diathesis will have a (mild) PFD of

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unidentified cause and will have a heterogeneous laboratory phenotype.[22] If a test with known limitations such as LTA would be considered a gold standard test in this study population, then new tests are likely to be nonsuperior because abnormal findings would then be considered false-positive although they might be accurate.

Genetic analysis could help the diagnosis of patients with inherited PFDs. The candidate list of genes involved in platelet function and thrombopoiesis currently includes as many as 329 genes,[64] which are expected to increase further in the future.[65] Nevertheless, a genetic abnormality is found in the minority patients with an identified PFD using laboratory testing.[66] Most of the causative genetic determinants of PFDs are still unknown, with exception of severe inherited platelet disorders such as GT or BSS. Genetic determinants of most other platelet disorders are less clear. Genetic analysis of patients with PFDs will hence yield genetic variants of unknown (even innocent) significance, and this will take years and extensive research to be validated.[67] Ultimately, this may allow prediction of bleeding severity based on a genetic profiling.[68] However, this will remain challenging in patients with bleeding disorders caused by multiple genetic abnormalities[69] or in those with mild PFD combined with other hemostatic disorders.[70] Unfortunately, genetic testing in acquired PFDs is not helpful, and therefore functional testing will remain always necessary.

An alternative to an observational design aimed to validate a diagnostic test is a (prospective) follow-up study design, in which outcome of the platelet function test under investigation is validated by association with future bleeding episodes in patients with suspected PFD. This design may be reliable in a cohort of patients with severe platelet disorders due to the frequent bleeding episodes they are likely to suffer. In case of mild PFDs, however, the bleeding episodes are rare, thus making it challenging to perform a follow-up study with sufficient power to draw conclusions.[71] Moreover, prophylactic treatment of (mild) bleeding disorders will preclude the use of a follow-up study design.[72]

Validation of a flow cytometry based platelet function test needs a clearly defined patient population with an elevated bleeding score. In these patients, coagulation factor deficiencies, VWD, and fibrinolysis disorders should be excluded. Preferably, disorders of vascular and connective tissue should also be ruled out, but this will be challenging. In this circumstance, the "gold standard" is the clinical presentation in combination with the exclusion of other easily diagnosed hemostatic disorders. By validating flow cytometry in this cohort, the diagnostic accuracy can be determined. The disadvantage of such a design is that the risk of false-positive results cannot be completely excluded, but this risk is likely to be similar for both the clinical presentation and flow cytometry. The number of false-positive results can be verified by repeated measurements. Moreover, perfect diagnostic accuracy is not predictable because the studied population may contain patients with normal platelet function, or the platelet function cannot be measured thoroughly with one static assay. Therefore, we should develop new tools providing added value to the currently available diagnostic workout for improving knowledge and detection of PFDs. This may especially encompass diagnostic tools allowing measurement of platelet adhesion function under flow conditions, which cannot be detected by either LTA or flow cytometry.

Conclusions

Platelets exert many different functions in the generation of a stable hemostatic plug. Different techniques are used for diagnosing PFDs in routine or specialized clinical laboratories, but they all have their own limitations. Diagnostics of PFDs might benefit from the use of flow cytometry because of its many practical advantages, its possible use in different disciplines, and the ability to assess a vast array of different platelet functions. Nevertheless, validation is necessary to establish its diagnostic value compared with the currently available tools and also for associating test outcome with clinical presentation. Enhanced diagnostic opportunities may improve the quality of life or treatment strategy and might ultimately increase the ability to dissect complex genotype–phenotype correlations.

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